Official journal website:
amphibian-reptile-conservation.org

Amphibian & Reptile Conservation
12(2) [Special Section]: 1–27 (e165).

Perspective

Integrating current methods for the preservation of amphibian genetic resources and living tissues to achieve best practices for species conservation

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***Abstract.*—Global amphibian declines associated with anthropogenic causes, climate change, and amphibian-specific infectious diseases (e.g., chytridiomycosis) have highlighted the importance of biobanking amphibian genetic material. Genetic resource collections were the first to centralize the long-term storage of samples for use in basic science, including disciplines such as molecular evolution, molecular genetics, phylogenetics, and systematics. Biobanks associated with conservation breeding programs put a special emphasis on the cryopreservation of living cells. These cell lines have a broader application, including the potential for genetic rescue and use in species propagation for population enhancement, such as captive breeding and reintroduction programs. We provide an overview of the most commonly used methods for the preservation of genetic resources, identify ways to standardize collection processes across biobanks, and provide decision trees to assist researchers in maximizing the potential use of their samples for both scientific research and the practice of species conservation. We hope that the collection and deposition of tissues preserved using methods that enable eventual cell line establishment will become routine practice among researchers, particularly herpetologists working in the field. While many major museums do not yet cryopreserve reproductive cells or cell lines, they contain the infrastructure and staff to maintain these collections if protocols and procedures are adapted. Collaboration between organizations can play an important future role in the conservation of amphibians, especially biobanks associated with research institutions and those pioneering techniques used in breeding programs.**

Keywords. ARTs, biobanks, cryopreservation, cell lines, tissue sampling, tissue culture, *in vitro* fertilization

Citation: Zimkus BM, Hassapakis CL, Houck ML. 2018. Integrating current methods for the preservation of amphibian genetic resources and living tissues to achieve best practices for species conservation. *Amphibian & Reptile Conservation* 12(2) [Special Section]: 1–27 (e165).

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Received: 30 November 2018; **Accepted:** 18 December 2018; **Published:** 31 December 2018

Global Amphibian Declines

With approximately one-third of all known amphibian species worldwide considered threatened, amphibians are currently the most threatened vertebrate group (Stuart et al. 2004; Wake and Vrendenberg 2008; Ceballos et al. 2015). Distressingly, these estimates do not take into account a large percentage of amphibians that are considered "data deficient" by the standards of the International Union for Conservation of Nature (IUCN 2018). The risk of underestimation is that even more species are threatened, especially in regions of the world that are known to be understudied (e.g., Madagascar, Southeast Asia; Rowley et al. 2010; Vieites et al. 2009). The hypothesized drivers of global amphibian decline include anthropogenic factors, such as habitat degradation or loss, overexploitation, pollution, and introduction of invasive species (Sodhi et al. 2008; Hof et. al 2011; Ficetola et al. 2014). Disease and climate change first emerged as the most commonly cited causes because almost 50% of amphibian species were characterized as having rapid and unexplained decline in areas where suitable habitat remained (Stuart et al. 2004).

Chytridiomycosis, an infectious disease in amphibians caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), is now known to be one of the proximate

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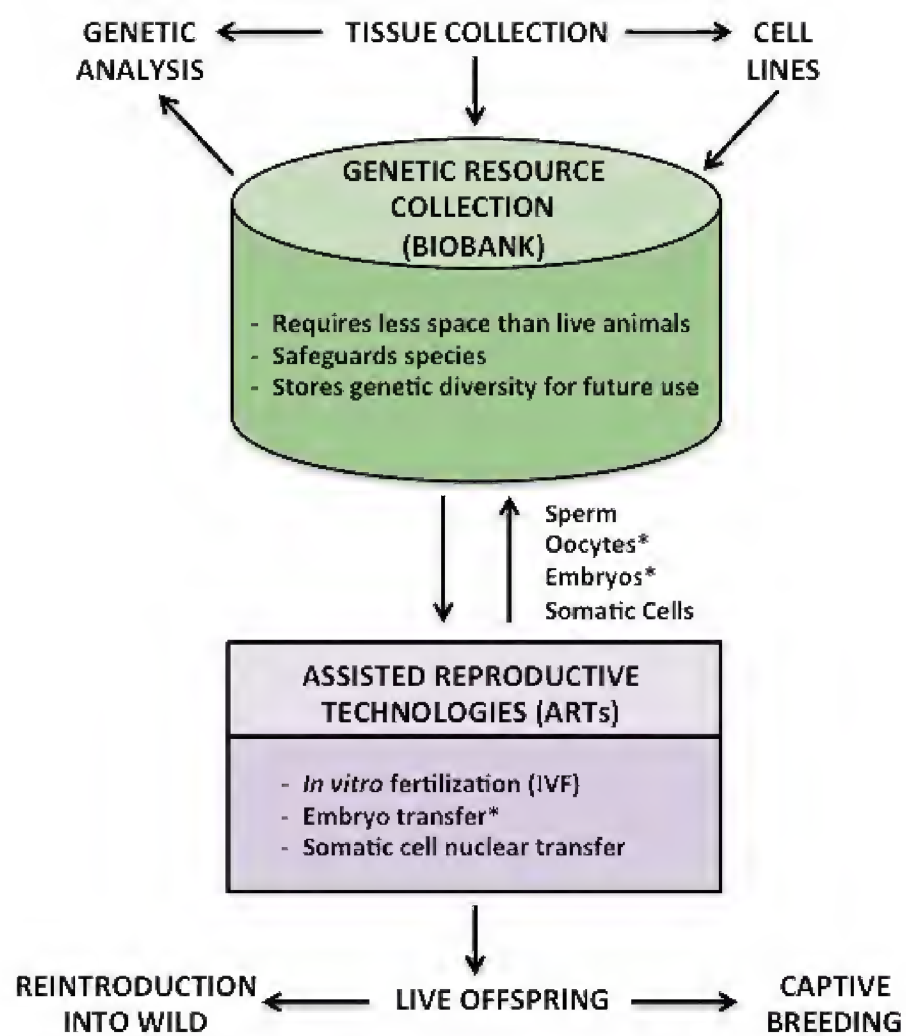

Fig. 1. Role of genetic resource collections in the research and conservation of amphibians. Green indicates the storage of tissues in biobanks. Purple indicates procedures associated with ARTs that lead to achieving multiple goals in amphibian research and conservation. Asterisk (*) denotes tissue or methodologies that are not currently used in ARTs but may be possible in the future. NOTE: For a more complete list of ARTs reference Clulow et al. (2014).

drivers of amphibian decline (Berger et al. 1998; Johnson 2006). The rapid decline of amphibians was linked with the emergence of *Bd*; the geographic ranges of declining species overlapped with areas most suitable for the fungus (Lötters et al. 2009). Zoospores produced by the fungus are dispersed in water and infect the keratin-containing epidermis in adults and the mouthparts in tadpoles (Berger et al. 2005). *Bd* has been documented to infect all extant orders of Amphibia and was detected in 41% of amphibian species across 63% of the countries in which sampling has been reported (Gower et al. 2013; Olson et al. 2013). A second species, *Batrachochytrium salamandrivorans* (*Bsal*), is known to cause the disease only in salamanders (Martel et al. 2013). Mitigating the effects of chytridiomycosis remains a major challenge, but data suggests that temperature range and precipitation may be of particular importance as the odds of *Bd* detection decrease with increasing temperature (Olson et al. 2013). In addition, recent work has found that increasing the salinity in aquatic habitats can block transmission and reduce the severity and mortality associated with *Bd*; hence, this tactic may be a promising focus for future management of this disease (Klop-Toker et al. 2017; Clulow et al. 2018).

Climate change has also been identified as a proximate cause of population declines because amphibians are sensitive to small changes in temperature and moisture given their permeable skin, biphasic lifestyle, and unshelled eggs (Pounds et al. 1999; Kiesecker et al. 2001; Carey and Alexander 2003). In addition, global warming has led some amphibians in temperate regions to breed earlier, making them vulnerable to early season freezes and floods induced by snowmelt (Beebee 1995; Blaustein et al. 2001; Gibbs and Breisch 2001). This trend was found to vary regionally for a single species, leading researchers to believe that climate change may be affecting amphibian populations in more subtle and complex ways. Hayes et al. (2010) suggested that interactions between multiple factors, including atmospheric change, environmental pollutants, habitat modification, invasive species, and pathogens, are the cause of amphibian declines. More recent work has found that although some amphibian communities are sensitive to changes in climate, observed declines can not be explained by the impact of climate change (Miller et al. 2018).

Regardless of the specific causes of global amphibian declines, there is an increased need to help prevent amphibian species extinction. One key approach to conservation is biobanking genetic resources of all types (e.g., somatic tissues, cell lines, gametes) before these resources are no longer available. We believe that integrating current methods used to preserve genetic resources and living tissues will facilitate stakeholder efforts and promote more effective cooperation to conserve amphibians (Hassapakis 2014).

History of Biobanking Amphibians

Biobanking, the practice of storing and curating genetic resources and their associated data, including cryopreserved living tissue, is one of numerous complementary methods that should be used to counteract global amphibian extinction and was included in the 2005 IUCN Global Amphibian Summit as one of 11 priorities relevant for amphibian conservation (Wren et al. 2015). Genetic resource collections form a critical basis for advances in scientific understanding of species (and species limits), evolutionary histories, and phylogenies. Many institutional biobanks that include amphibian genetic resources are associated with natural history museums (e.g., Harvard's Museum of Comparative Zoology, Smithsonian's National Museum of Natural History), while laboratories or departments within colleges/universities (e.g., Kansas University Biodiversity Institute, The University of Texas at El Paso) have also become *de facto* biobanks because individual researchers have amassed large and/or important sample collections (Zimkus and Ford 2014b). Amphibian genetic resources are also essential for conservation breeding programs (CBPs; species propagation for population enhancement), which store primary cell cultures for current and future use in habitat restoration, reintroduction from captivity to the wild, and captive management (Fig. 1).

Tissue samples traditionally collected and used in phylogenetic and systematic studies have long aided in understanding global amphibian diversity, resolving phy-

logenetic relationships for closely-related species and revealing cryptic species that were morphologically indistinguishable (Fouquet et al. 2007; Vieites et al. 2009). Species delimitation is needed for population assessments and assists in the understanding of species ranges, and accurate taxonomic assignments allow the identification of characteristics (e.g., endemic species/lineage, population declines, threatened species) informative for assisting in conservation assessments and priorities (Mace 2004). In addition, modern molecular approaches have allowed a greater understanding of amphibian declines, characterizing the prevalence of infectious diseases and assessing the effects of habitat alteration on population connectivity (Storfer et al. 2009). Therefore, genetic vouchers from newly described species, especially if there are limited voucher specimens or the species has a highly restricted distribution, should be deposited in established biobanks for future conservation options (García-Castillo et al. 2018).

Those collecting amphibian specimens for molecular analysis have known for decades that the traditional preparations used primarily for morphological studies (e.g., fixation in formalin) are not ideal for DNA sequencing protocols because these methods induce DNA interstrand crosslinks, cause base modifications, and induce fragmentation (Campos and Gilbert 2012; Do and Dobrovic 2012; Quach et al. 2004; Wong et al. 2014). Procedures, therefore, evolved to include sub-sampling of tissues before specimens were exposed to formalin and low-concentration ethanol, thereby avoiding extensive DNA damage. Many institutions found the value in the genetic resources collected for project-based purposes and began to curate these collections for long-term preservation, including the formation of centralized biobanks (Zimkus and Ford 2014b). Although these genetic resources were likely collected for specific purposes associated with the original research, it was clear that samples could be used in future studies, making it possible for others to avoid costly and time-consuming fieldwork required to collect new samples (Astrin et al. 2013). In addition, institutions realized that the utility and value of samples may actually increase as rapidly-changing technology and newly-developed methods allow samples to be used in ways that are not currently possible.

The primary goals of amphibian conservation breeding programs include building genetically representative captive populations, and maintaining the health, reliable reproduction, and perpetuation of genetic variation. Storage of genetic material in the case of amphibians is important insurance against possible extinction and can be used to reduce the loss of genetic diversity in captive colonies and in declining wild populations (Fig. 1). Some biobanks house samples for purposes of species propagation using Assisted Reproductive Technologies (ARTs), including gamete cryopreservation and *in vitro* fertilization (IVF), where resulting offspring may be used in captive breeding or reintroduction programs. A number of these types of biobanks exist, including the Memphis Zoo (Department of Research/Conservation) and San Diego Zoo Global in the United States (Frozen Zoo®, San Diego Zoo Institute for Conservation Research), the Zoological Society of London, the Institute of Cell Biophysics at the Russian Academy of Sciences in Moscow, and the University of Newcastle in Australia (Kouba and Vance 2009). The rapid loss of amphibian species has more recently resulted in the World Associations of Zoos and Aquariums (WAZA) promoting the formation of conservation breeding programs supported by research as a key element of their conservation plans (WAZA 2005), which has likely led to an increase in the number of biobanks in recent years. Kouba et al. (2013) report that biobanks are being initiated or planned at the Smithsonian Conservation Biology Institute in the U.S., the Toronto Zoo in Canada, the New Zealand Centre for Conservation Medicine at Auckland Zoo, and the University of Wollongong in Australia.

Considerations for Preservation

Aims and Goals

Numerous methods are currently being used to preserve amphibian tissue and likely depend on the specific aim of a scientific study or goals of an institutional or collaborative program (e.g., biobank, multi-institution initiative). Samples may be collected for individual research projects with explicit and relatively short-term goals (e.g., molecular ecology, molecular phylogeny, population genetics). Studies may also be taxonomically or regionally-focused, such as rapid biodiversity assessments that use DNA barcoding techniques to identify species surveyed in a specific region. In contrast, biobanking initiatives or collaborative programs involving multiple institutions may have targeted specific species for long-term conservation and/or use in ARTs. The ultimate aim of a research study or conservation initiative may dictate the specific tissue types or biomolecules needed to fulfill the project goals. Molecular studies traditionally used DNA as it could be preserved more easily in the field with many methods. Unfortunately, the various methods used for DNA preservation are not equally effective, and DNA may be fragmented or otherwise compromised. Some preparations may allow high-quality Sanger sequencing reads but prevent high-quality gDNA needed to sequence genomes or the high-molecular-weight DNA needed for long-read sequencing and other technologies (e.g., BAC library preparation, optical mapping, 10X Chromium libraries; Mayjonade et al. 2016). RNA is increasingly being used in gene expression studies but is preserved using fewer methods and degrades rapidly if not frozen immediately. Researchers should, therefore, consider all preservation options as some may allow them to both fulfill their study goals and aid in current or future research or conservation initiatives.

Table 1. Tissue types commonly used for genetic study, growth of cell lines, and ARTs in amphibians. Preferred tissues for the production of cell lines are included in parentheses, although other tissue types that have been successful are listed. NOTE: Asterisk (*) denotes unsuccesful efforts to cryopreserve to date (Clulow and Clulow 2016).

	Preserve for Genetic Study	Make Cell Lines	Make Cell Lines in Future	Collect and Use Immediately in ARTs	Preserve for Future Use in ARTs
Testes	X	(X)	(X)	X	X
Ovaries	X	(X)	(X)	X	
Limb/foot	X	(X)	(X)		
Skin (Biopsy)	X	(X)	(X)		
Tongue	X	(X)	(X)		
Eye	X	X	X		
Kidney	X	X	X		
Tadpole	X	X	X		
Tail clip	X	X	X		
Lung	X	X			
Toe clip	X	X (if large)			
Embryos	X	X			*
Spermic urine	X			X	X
Sperm	X				X
Blood	X				
Feces	X				
Glands	X				
Heart	X				
Liver	X				
Muscle	X				
Oocytes	X				*
Pancreas	X				
Spleen	X				
Swab (e.g., skin, mouth)	X				

Tissue Types

Many different tissue types can be preserved for use in basic genetic studies as many soft tissues yield high-molecular-weight-genomic DNA (Table 1). Liver and skeletal muscle are perhaps the most commonly sampled tissues for herpetological research (Gamble 2014). A small incision can allow researchers to push the liver out, causing minimal damage to specimens being used for morphological study. Camacho-Sanchez (2013) also found that rat (*Rattus rattus*) liver yielded the best RNA and DNA quality when compared to blood, brain, ear clips, muscle, and tail tips. Although liver is widely used by those collecting amphibian genetic samples, bile salt can contaminate this organ and affect tissue stability, so tissue should be preserved as soon as possible and the gallbladder avoided (Dessauer et al. 1990). Muscle can be dissected from the thigh on one side, leaving the remaining side intact for morphology, but it has been reported that yields are small due to tough fibers (Gamble 2014; Wong et al. 2012). According to Wong et al. (2012), testes provide high yields and are the preferred tissue in species with heterogametic or temperature-dependent sex determination, while liver is recommended for immature specimens and homogametic individuals (females in XY, males in ZW systems). Others recommend sequencing genomes from the heterogametic sex or both sexes for amphibians as this may provide important information about sex determination in different species, which is relevant for managing captive populations and reproduction (Tony Gamble, pers. comm.). Wong et al. (2012) notes that soft tissues (e.g., spleen, pancreas, lung, glands) are prone to faster degradation, so harder tissues (e.g., muscle, kidney, heart) may be preferable. Lastly, Wong et al. (2012) suggest that red blood cells are a good source of high-molecular-weight DNA and are the preferred tissue for constructing large-insert libraries and for use in long-read sequencing. Blood collection may be difficult for small species, but techniques using doppler ultrasound and fiber-optic lights may make it more feasible (Gamble 2014).

The collection of samples from different tissue types (stored in separate vials) is desirable for RNA studies, achieving the highest possible coverage of the diverse

transcriptome, as well as optimizing the chance of establishing a successful cell line. Contractile proteins, connective tissue, and collagen in skeletal muscle, heart, and skin tissue may result in low RNA yield (Wong et al. 2012). For cell lines, the recommended tissues include (in order from most to least successful): whole limb (i.e., foot), tongue, skin, and gonads (Table 1). Viable cell lines provide the highest quality material for DNA and RNA, and additionally can be used for chromosome analysis and potentially reprogrammed into induced pluripotent stem cells (Takahashi et al. 2007; Yu et al. 2007; Ben-Nun et al. 2011) that can differentiate into any type of cell, including gametes. Species propagation in amphibians historically requires reproductive cells (e.g., sperm, oocytes) for ARTs and has proven successful with the use of testes and ovaries (Table 1).

Destructive vs. Non-Destructive Sampling

Tissue samples traditionally collected and used in phylogenetic and systematic studies are often associated with whole-animal voucher specimens deposited in natural history collections. Recently-deceased animals are also a source of both tissues that can be used for genetic study and material used in species propagation. A number of factors may result in the choice of less destructive protocols associated with sample collection. For example, projects may require links between genetic samples and live animal 'vouchers' in zoos, aquaria, universities, and other institutions (e.g., CryoArks project; U.K. Research and Innovation 2018). Collection of samples from live animals require less invasive methods that do not affect the animal's fitness and precludes the collection of vital organs, such as the liver, that require animal euthanization. Non-lethal sampling methods may include biopsies, blood draws, feces collection, skin swabs, sperm or spawn collection (hormonally-induced), toe clips, and tail clips (Ezaz et al. 2009; Gamble 2014; Mollard 2018; Mollard et al. 2018). The feasibility of obtaining sperm and eggs in the field and laboratory has been demonstrated both in frogs and salamanders (Shishova et al. 2011; Figiel Jr 2013; Uteshev et al. 2013; Uteshev et al. 2015). Non-invasive sampling methods for use in genetic analyses, including the detection of chytrid fungus using skin swabs, is becoming increasingly common with amphibians (Pichlmüller et al. 2013; Soto-Azat et al. 2009). Certain specimens, such as those designated as type material, may require similar sample collection methods that minimize external damage to retain all parts needed for taxonomic diagnosis. Small animals or early developmental stages may have low amounts of tissue available, and hence eggs, tadpoles, metamorphs or juveniles may need to be collected whole.

Collection of Gametes

Timing of tissue collection should be synchronized with breeding season if the goal is the collection of gametes for ARTs. Whenever possible, reproductively active animals should be collected during or close to breeding season, which makes obtaining gametes (primarily sperm) much less laborious (Childress 2017). Collecting animals out-of-season or not of reproductive age requires that animals be maintained in captivity until gametes can be collected naturally or breeding is induced through hormone usage. Housing and monitoring the reproductive status of animals requires veterinary permits, substantial time, skill in captive husbandry methods, and species-specific nutrition, as well as social and behavioral specifications. Monitoring live animals can become time-consuming and complex when multiple species have different diets and housing requirements. In addition, many anurans do not reproduce easily in captivity because of confinement stress or lack of critical environmental cues needed to induce reproduction (Kouba et al. 2009). Given that the most time-consuming aspect of collecting gametes for cryopreservation is the timing of natural reproduction, amphibians can be injected with hormones to induce spawning and reduce required time in captivity (Fig. 2; Rugh 1934; Miller 1985; Browne et al. 2006; Trudeau et al. 2010; Trudeau et al. 2013).

Logistics

Researchers collecting samples need to consider the location of initial preservation and if possible carry out a feasibility study to ensure that the selected preservation method(s) will work given any logistical constraints. Those transporting live animals to permanent laboratories for sample collection or using mobile labs have the most choices in regards to preservation methods. Working within a short distance from the laboratory or biobank requires the development of field protocols that adapt laboratory techniques given local conditions at the collection site but offers numerous options for sample preservation. In contrast, fewer methods allow samples to be collected and transported from remote locations for a number of reasons: 1) the preservatives or equipment needed to maintain the samples may not be available in the specific country or collection site, 2) the duration of the field trip or time required to transport the samples may eliminate specific methods, and 3) the ambient temperature at the collection site or temperatures that the samples are exposed to during transit may preclude use of specific methods.

Shipping biological materials requires attention to the type of material transported, adherence to regulatory requirements, packaging materials and proper assembly, labeling, and engaging reputable carriers (Simione and Sharp 2017). International shipments that include dangerous goods must follow International Air Transport Association (IATA) Dangerous Goods Regulations to meet commercial standards, while domestic shipments must follow national guidelines. Legal requirements as-

sociated with the transportation of dangerous goods or hazardous materials may preclude the shipping of some preservatives either using a courier or in personal airline baggage; therefore, shipping options should be determined given the materials (e.g., infectious agents), preservatives (e.g., hazardous chemicals), and cold-chain methods (e.g., dry ice, liquid nitrogen [LN_2]) employed. Courier services that maintain samples at required temperatures can be considered for viable material but are expensive.

Ethical and Legal Requirements

Scientific procedures carried out on animals should minimize adverse effects while maximizing the scientific benefit gained. These legal and ethical requirement are included under the laws and regulations of numerous countries worldwide, including the U.S. Animal Welfare Act (United States Code, Title 7, Chapter 54, Sections 2131–2159), the U.K. Animals (Scientific Procedures) Act 1986, the U.K. Animal Welfare Act 2006, the Animal Health and Welfare Strategy for Great Britain, Animal Welfare Strategy, Canadian Council on Animal Care in Science, among others. Researchers should be aware of laws and regulations associated with their home country and possibly the country of origin of the specimens collected. Within the U.S., an Institutional Animal Care and Use Committee (IACUC) ensures that all projects involving the use of live vertebrate animals comply with federal regulations and guidelines (OLAW/ARENA 2002). An IACUC is required by federal regulations for most institutions that use animals in research, teaching, and testing and has a key oversight role, including the review and approval of animal use activities. IACUC review of such studies would focus on, but not necessarily be restricted to, such issues as: number of animals to be used in a study; stability of the population from which the animals are to be taken; appropriateness of the methods used for capturing, immobilizing, and/or euthanizing animals; and training and supervision of the personnel involved with the study. To this end, both collection procedures and animal husbandry practices must be planned in advance and approved to meet the intended goals and objectives of the research project.

Proper planning for collection of specimens/samples includes researching the permits needed to conduct research, collect, and export scientific specimens from a specific country. The Nagoya Protocol on Access and Benefit-Sharing (ABS) is (for its contracting parties) a legally binding supplementary agreement to the Convention on Biological Diversity (CBD) that affirms that countries hold sovereign rights over their biological resources. Those collecting genetic samples should, therefore, determine country-specific permitting requirements using the ABS Clearing-House (Secretariat of the Convention on Biological Diversity 2018), including obtaining *Prior Informed Consent* (e.g., collecting permit) from the providing country and establishing *Mutually Agreed Terms* (e.g., benefit-sharing agreement) if needed. In some countries separate permits may be required for collecting wildlife and taking genetic resources. In addition to national permits, other permissions and documentation may be needed to research and/or collect particular species or in specific regions (i.e., protected lands), as well as import specimens into the destination country. Lastly, indigenous communities may have legal authority over wildlife and may have requirements associated with collecting materials (e.g., New Zealand). Given that the process of applying for and receiving permission to conduct research and collect specimens may take substantial time, permits and any other required documentation should be secured as far in advance as possible to alleviate complications that might slow or jeopardize research projects. For those working internationally, collaboration with in-country partners (e.g., local scientists, wildlife managers) should be considered as it may facilitate the permit process and fulfill benefit-sharing obligations.

Best Practices in Tissue Preservation for Genetic Analyses

Tissue preservation (fixation) methods used for amphibian samples generally prevent or reduce enzymatic and thermodynamic degradation of nucleic acids (Yagi et al. 1996; Prendini et al. 2002). A review of tissue preservation methods for use in molecular studies was first presented by Prendini et al. (2002) and later updated by Nagy (2010). In addition, Gamble (2014) provided information specific to collecting and preserving genetic material from herpetological specimens. These reviews provided thorough overviews of tissue preservation methods for molecular genetic analyses, outlining the advantages and disadvantages of each method. A global survey of 45 independent genetic resource collections within 39 different institutions found that the vast majority (80%) of genetic resource collections store samples that were initially preserved in multiple (2–5) different ways, which is expected given that the majority of these collections stored samples collected for individual research projects (Zimkus and Ford 2014). This survey included numerous types of institutions and was not taxonomically-focused, but over two-thirds (64%) of the respondents reported that their collections stored amphibian samples. Data from the 29 collections reporting amphibian genetic resources was analyzed for this study to determine the most commonly used procedures associated with initial preservation in an attempt to provide more accurate statistics; however, it should be noted that most of these collections house diverse taxonomic collections, so responses may also be applicable to non-amphibian collections.

All of the genetic resource collections that included amphibian samples indicated that they housed samples preserved with 95–99% ethanol with two noting that most or all samples were preserved in 99% ethanol. Over

three-fourths of collections (77%) reported that samples were initially flash-frozen at a temperature of -80 °C or below; however, the survey question did not ask respondents to clarify the technique used: frozen on dry ice (at -78.5 °C), mechanical freezers (-80 °C to -150 °C), immersion in LN_2 (-196 °C), or storage in the vapor-phase of nitrogen (≤-150 °C). Respondents also reported their collections included samples initially preserved in dimethylsulfoxide (DMSO; 45%), which is a commonly used cryoprotectant, but the survey did not ask respondents to specify the temperature at which the preservative was used. Fewer collections used commercial products, such as RNA*later*® (Ambion; 52%) or *Allprotect*® Tissue Reagent (Qiagen; 10%) as an initial preservative; however, non-hazardous proprietary agents that allow the preservation of multiple biomolecules at ambient temperature are being increasingly used (Muller et al. 2016). Herein we will discuss these most commonly used methods of sample preservation identified for collections biobanking amphibian genetic resources, addressing the advantages and disadvantages for both researchers making the initial collections and biobankers concerned with long-term storage and downstream use.

Freezing

One of most effective techniques for the long-term stabilization of genetic samples is cryopreservation, an approach that is based on the principle that cryogenic temperatures suspend biological, chemical, and physical processes (Karlsson and Toner 1996). Depending on the temperature, freezing samples reduces or inhibits the enzymatic and chemical activity that leads to nucleic acid damage and degradation (Engstrom et al. 1990; Karlsson and Toner 1996). Degradation is virtually absent if samples are continuously kept in LN_2 storage (generally between -150 °C and -196 °C, depending on whether vapor-phase or liquid is used) because of lack of thermal energy needed for chemical reactions; however, freezing damage, including cell death, can be caused by ice formation and crystallization if cryoprotectants are not used (Karlsson and Toner 1996). Therefore, the initial preservation method, any subsequent methods used in long-term storage, and all downstream handling (e.g., freeze-thaw events, changes to preservative) will affect the quality of the sample (Benson et al. 2016).

Many methods use freezing or flash-freezing as one component to preserve genetic samples. Freezing samples without the use of a preservation agent either by placing them into a laboratory freezer (≈-20 °C) or by using ice within an insulated container is generally not recommended because temperatures are not low enough to prevent enzymatic activity nor the formation of intracellular ice crystals (Stoycheva et al. 2007; Nagy 2010). Those collecting samples may have access to mechanical freezers that can maintain samples at ultracold or cryogenic temperatures (-80 °C to -150 °C), but this generally excludes samples that are collected in the field. Dry ice (frozen carbon dioxide; -78.5 °C) stored within insulated containers may allow the collection or transport of samples, but a sublimation rate of approximately 10% or 2–5 kg every 24 hours limits this method to relatively short trips. In addition, since temperatures near the upper end of the ultra-low temperature range, some DNA degradation may occur as a result of weak enzymatic activity (Nagy 2010).

Cryogenic storage dewars, a specialized type of vacuum flask used to store cryogenic fluids, can be used to flash-freeze samples in LN_2. Access to LN_2 increases field sample collection options, including the preservation of tissues useful for cell culture, RNA, and gametes for multiple weeks. If a LN_2 source is available, samples can be kept viable in the field until they can be transported back to a laboratory. The use of LN_2 requires additional precautions as it can cause frostbite, cold burns, and asphyxiation by displacing the oxygen of the surrounding area. In addition, sample vials can shatter when removed from storage because LN_2 can enter the vials and rapidly expand upon warming, creating a hazard from both flying debris and exposure to the contents. Secondary containment (e.g., polyethylene tubing, tin foil) and protective eyewear is, therefore, recommended. Cross-contamination has been reported for samples immersed directly in LN_2, so researchers should consider vial type, secondary containment options, and use of vapor-phase storage when considering flash-freezing methods (Clark 1999).

Freezing without the inclusion of a preservative was once thought to maximize future research potential, but data now suggests that buffered samples or those stored in a cryoprotectant, such as DMSO or glycerol, may perform better after thawing and refreezing (Nagy 2010). Cryoprotectants partly protect against degradation occurring during temperature changes, such as freeze-thaw cycles. Mulcahy et al. (2016) found that fish tissues stored in a solution with 25% DMSO or DNAzol yielded higher quality DNA after thawing than putting tissue directly into LN_2 or -20 °C storage without buffer, while crab tissues in DMSO and ethanol did equally well in preserving DNA quality. If viable cells are desired (e.g., cell culture, gametes), slow freezing and using a cryoprotectant is required to prevent the formation of ice crystals that lead to fatal cell lysis.

A dry shipper is an insulated cryogenic flask/container that contains LN_2 absorbed into a porous lining. Dry shippers are not considered a dangerous product and hence can be used to ship samples by plane if the liquid is fully absorbed and excess poured off. There are wide variations in dry shippers with regard to size and temperature (static) hold times. Size ranges include those with space for a dozen vials to others that can accommodate thousands of vials. Temperature hold times can vary from a few days to multiple weeks, and variations also exist in how well they hold temperature under dif-

ferent environmental and handling conditions. Although dry shippers are expensive to purchase, they can be used for many years. Performance degradation can be attributed to catastrophic or gradual vacuum loss of the dewar, accumulation of moisture in the LN_2 absorbent material, or damage and loss of portions of the absorbent material (Simione and Sharp 2017). It is recommended that the performance of dry-shipping dewars be checked at regular intervals and ideally before each use using a simple 24-hour evaporation test to identify whether there has been deterioration in any components.

An increasing number of biobanks associated with natural history collections are using LN_2 cryovats for long-term storage of samples. Zimkus and Ford (2014b) surveyed genetic resource collections associated with natural history museums and found that vapor-phase nitrogen storage that include a standing level of liquid below a rotating carousel was the most commonly used type of cryovat. Those managing collections should be aware of the previously-discussed safety issues associated with both cryogenic liquids and vials previously immersed in LN_2; for additional information about proper ventilation and use of oxygen monitors in genetic resource collections with LN_2 storage, see Zimkus and Ford (2014a).

Ethyl Alcohol (Ethanol)

Alcohols, and specifically ethanol, are the most frequently used chemical to preserve amphibian tissues. Ethanol denatures proteins that may degrade DNA and is able to preserve samples for long periods of time at ambient temperature; RNA cannot be preserved using ethanol at room temperature. Survey data indicated that all participating genetic resource collections stored samples preserved in 95–99% ethanol (Zimkus and Ford 2014b). Ethanol concentration can greatly affect the resulting quality of the samples with 95–96% (190 proof) recommended as optimal. In most countries 190 proof ethanol is widely available for purchase at pharmacies. Concentrations above 96% (including absolute ethanol) are not recommended as they likely contain traces of drying agents (e.g., benzene) that can affect DNA preservation (Ito 1992). Concentrations of 65–75% (commonly used to preserve whole animals for morphology) are also not recommended; Seutin et al. (1991) were unable to recover DNA from bird brain and muscle samples kept in 70% ethanol for six weeks at room temperature, while liver samples yielded significantly degraded DNA. Researchers should avoid using distilled alcoholic beverages because they may have alcohol concentrations as low as 35%. Undiluted rectified spirits or neutral spirits (e.g., Everclear, Crystal Clear, Primasprit, Spirytus) is highly concentrated (95–96%) but should be avoided because it includes denaturing chemicals. Denatured alcohol (i.e., methylated spirits), widely used for industrial purposes, is made of 70–99% ethanol but contains additives that make it non-consumable for humans (e.g., methyl ethyl ketone, also known as MEK) and thus should also be avoided (Post et al. 1993; Dillon et al. 1996).

Using ethanol as a preservative has numerous advantages for researchers whose primary goal is to preserve DNA. Ethanol is easy to use and able to preserve DNA even in areas with elevated ambient temperatures for long periods of time, although the liquid is flammable and considered hazardous. Nagy (2010) suggested that tissues be cut into small pieces to increase the surface area, using at least 5:1 volumes of ethanol, while others suggest higher ratios (Martin 1977). Although the initial concentration and ratio of ethanol to sample is important, changing the alcohol during the first one to two days of storage is also necessary because samples release water and progressively dilute the preservative (Kilpatrick 2002; Nagy 2010). Researchers should be aware that most inks are soluble in ethanol, so pens used for labeling vials containing ethanol should be tested before use. A secondary labeling method should also be considered for redundancy, such as labeling with graphite pencil, engraving or barcoding. Placing paper tags inside vials has been used as a method of labeling, but it is currently unknown whether this may lead to contamination (Zimkus and Ford 2014b).

The transport of non-infectious ethanol-preserved specimens has been allowed since 2011 via International Air Transport Association (IATA) Special Provision A180, making it possible to transport specimens preserved in ethanol. The following packing and marking requirements must be met, including: 1) specimens are placed in vials or other rigid containers with no more than 30 ml of alcohol or an alcohol solution; 2) the specimens are then placed in a plastic bag that is then heat-sealed; 3) the bagged specimens are then placed inside another plastic bag with absorbent material then heat-sealed; 4) the finished bag is then placed in a strong outer packaging with suitable cushioning material; 5) the total quantity of flammable liquid per outer packaging must not exceed one L; and 6) the words "scientific research specimens, not restricted, Special Provision A180 applies" must be written on both the outside of the package and on the air waybill in the description of the substance.

Those maintaining archival collections, including biobanks, can combine initial preservation in ethanol with long-term cold storage at cryogenic temperatures to preserve DNA indefinitely. Since the melting/freezing point of pure ethanol is approximately -114 °C (-173 °F), high-concentration ethanol thaws almost immediately after removing from LN_2 storage. Procedures associated with sub-sampling can be completed significantly faster when the storage medium does not require thawing. Nucleic acids are sequentially degraded by cycles of freezing and thawing, but anecdotal evidence suggests that samples can be thawed and refrozen several times (Shao et al. 2012). Collections storing samples in vials with a silicone O-ring should be aware that, according to the manufacturers (e.g., Nalgene, NUNC), these types

of vials were designed for tissue culture and are vapor permeable. After observing that rapid evaporation was occurring in their ethanol-preserved tissue collections on a scale of weeks, the North Carolina Museum of Natural Sciences conducted tests and found that 95–100% ethanol evaporated fastest at room temperature and slowest at -80 °C (Bryan Stuart, pers. comm.).

Dimethyl Sulfoxide (DMSO)

DMSO is commonly used in aqueous solutions to preserve DNA as it readily permeates tissues and enhances absorption of materials that inhibit nucleases (e.g., EDTA, NaCl). In addition, like glycerol, DMSO prevents cellular damage from formation of ice crystals, making it an effective cryoprotectant. A number of solutions with 20–25% DMSO, 0.25 M disodium-EDTA, and salt to saturation have been shown to be effective (Dawson et al. 1998; Kilpatrick 2002; Seutin et al. 1991). Kilpatrick (2002) found that a 3:1 DMSO-salt solution provided the best protection from DNA degradation of mammalian liver tissues stored for up to two years when compared to 95% ethanol and lysis buffer. Nagy (2010) recommended that the ratio between DMSO and sample exceed 5:1 but at the very least be 3:1 for effective preservation. These cost-efficient solutions can be easily made in the laboratory, are associated with only minor health concerns (e.g., skin irritation), and can be shipped without restrictions (Kilpatrick 2002; Nagy 2010).

Although DMSO-salt solutions are an effective preservation method, there are a number of drawbacks. One major issue is that these solutions preserve DNA and not RNA at room temperature. Although these solutions have been well-tested with marine invertebrates and mammals, only anecdotal evidence seems to exist regarding its effectiveness in the preservation of amphibian tissues. In addition, there have been no long-term studies to test effects on tissue and DNA quality over periods of time relevant to museum collections. For those working with archival samples, tissue can become encrusted with salt, making it more difficult to sub-sample. In addition, it may be toxic at high levels to living cells.

Commercial Products for Ambient Storage

A number of proprietary products are available for ambient temperature stabilization with increasingly more products available every year (Muller et al. 2016). RNA*later*® is commonly used by researchers depositing their samples in genetic resource collections associated with natural history museums (Zimkus and Ford 2014). RNA*later* is an aqueous, nontoxic tissue storage reagent marketed to preserve RNA up to one day at 37 °C, up to one week at 25 °C, up to one month at 4 °C, and indefinitely at temperatures of -20 °C or below. According to both the manufacturer and published studies, RNA*later* has been tested and found to be successful in preserving many animal tissues (e.g., brain, heart, kidney, spleen, liver, testis, skeletal muscle, fat, lung, and thymus; Nagy 2010; Camacho-Sanchez 2013). According to the manufacturer, this product is not recommended for bone because of the lack of sufficient penetration into the tissue. In addition, use of RNA*later* to preserve RNA in blood and plasma have more involved procedures.

RNA*later* should only be used with fresh tissue and requires that samples be cut into small pieces (i.e., less than 0.5 cm in one dimension) and placed in 5–10 volumes of the solution. Previously frozen tissues thaw too slowly in RNA*later*, preventing the reagent from diffusing into the tissues quickly enough to prevent nucleic acid degradation. It is recommended that tissues are incubated overnight at 4 °C to allow thorough penetration; however, if ambient temperature is above 25 °C, it is suggested that samples are placed on ice for a few hours after being placed in RNA*later* before storing at ambient temperature.

For researchers making initial collections, there are a number of strengths associated with this product, including this single solution is able to stabilize and protect both RNA and DNA at ambient temperature. In addition, RNA*later* is not considered hazardous for shipping, making it easy to transport to and from field collection sites. This product does have a number of limitations that may make it difficult to use for researchers at remote sites, in areas where the ambient temperature is above 25 °C (unless refrigeration is available and cold-chain can be maintained during transport), and long duration trips. This product is considered expensive (e.g., $348 US/250 ml), but researchers have devised homemade versions that may be more cost-effective, although their efficacy is yet untested (Nagy 2010).

If the product is used according to the manufacturer's recommendations, samples initially preserved in RNA*later* and stored in biobanks or for archival purposes can be used to extract both RNA and DNA. In addition, samples can be thawed at room temperature and refrozen without significantly affecting the amount or integrity of recoverable RNA or DNA. It is recommended that samples be removed from the solution before long-term storage because the liquid requires substantial time to thaw and expands upon freezing, so overfilled vials may crack or explode. There have yet been no long-term studies to test effects on tissue and sample quality over periods of time relevant to museum collections (i.e., decades).

AllProtect® has also been used to preserve samples deposited in genetic resource collections, although less frequently compared to RNA*later* (Zimkus and Ford 2014b). This gel-like tissue storage reagent preserves DNA, RNA, and proteins for up to one day at 37 °C, at room temperature (15–25 °C) for seven days, 2–8 °C for up to six months, or indefinitely below -20 °C. The reagent is provided with a pump that dispenses approximately 0.5 ml at a time; other methods of aliquoting may be

difficult because of the viscosity of the reagent. Fewer studies have been completed compared to RNA*later*, but *AllProtect* is recommended by the manufacturer for most animal tissues, excluding bone because of lack of penetration, and it is not suitable for stabilizing cultured cells, whole blood, plasma, or serum. Similar to RNA*later*, fresh tissues (not previously frozen) must be cut into small pieces (less than 0.5 cm in one dimension), and samples must be placed in at least 10 volumes of solution.

The strengths of *AllProtect* are associated with the fact that it allows preservation of multiple biomolecules at ambient temperature and is not considered hazardous when shipping. The reagent is viscous and maybe more difficult to use when compared to RNA*later*. *AllProtect* is considered very expensive (e.g., $645 US/100 ml), almost five times more expensive when compared by volume to RNA*later*, which is already cost-prohibitive for some researchers. In addition, the reagent is only stable for six months after the product is open, so it would need to be purchased each year for annual fieldwork. The manufacturer reports that RNA remains intact up to 15 freeze-thaw cycles, while proteins remain intact for five freeze-thaw cycles, which is beneficial for biobankers who may be sub-sampling a specimen numerous times for different requests. The manufacturer does recommend that excess product is removed from the sample surface (e.g., dabbing, rolling over paper towel) before long-term storage, which may require substantial time for biobankers. In addition, no long-term studies have tested the effects on tissue and DNA quality over periods of time relevant to museum collections.

Best Practices for Gamete Preservation for Use in ARTs

Gonadotropic Hormones

ARTs for amphibians are based on the use of gonadotropic hormones (e.g., hCG, synthetic analogs of luteinizing hormone releasing hormone [LHRH]) to trigger spawning and the maturation of gametes for collection, cryopreservation, and potential future use in artificial fertilization (Ananjeva et al. 2017; Norris and Lopez 2011). Gonadotropic hormones are injected to stimulate natural reproductive and spawning behavior in amphibians and are most often used outside of the natural breeding cycle (Goncharov et al. 1989). Experimentally it has been shown in frogs and salamanders that these general protocols induce reproductive behaviors (e.g., amplexus, deposition of eggs) in amphibians (Kouba et al. 2009; Kouba and Vance 2013; Vu and Trudeau 2016). A current review of Australian frogs reports that usage of gonadotropic hormones (single dose of gonadotropic-releasing hormone [GnRH] or hCG) is effective in Myobatrachidae and Limnodynastidae for induction of spermiation and ovulation (Clulow et al. 2018a) but remains much more problematic for the Pelodryadidae group of species. Hormones are used to obtain mature gametes for immediate, postponed (i.e., days), or suspended (i.e., months, years) artificial fertilization (Ananjeva et al. 2017).

Frog Sperm Collection and Preservation

Anuran sperm from many species has been shown to remain viable (defined by motility or membrane integrity) when refrigerated from days to weeks. Ultimately, the production of offspring helps to maintain conservation breeding populations in captivity, bolster natural populations, and reintroduce populations into areas where it has been extirpated. If there are no immediate plans to breed frogs, cryopreserving sperm allows for future attempts at species propagation using ARTs, collecting these valuable resources while they still exist in nature and are relatively easy to collect, archiving in biobanks and prioritizing endangered and threatened species and species from highly threatened and endangered habitats.

Sperm can be sampled from deceased frogs by macerating excised testes and using physiological solution to prevent activation. Sperm motility depends on solution osmolarity with initial activation occurring in hypotonic solutions below 250 mOsmol/kg with respect to blood plasma; most sperm are activated between 100–50 mOsmol/kg, and total activation occurs in solutions with osmolarities of approximately 50 mOsmol/kg (Ananjeva et al. 2017). Testicular sperm can be cryopreserved and used at a later date using sperm collected by maceration of testes or by the more recent and non-lethal method of hormonally induced sperm (HIS) collected in urine (Browne et al. 1998; Shishova et al. 2011; Uteshev et al. 2013). Physiological modifications to be considered for amphibian sperm cryopreservation are related to extracellular osmolarity variation, effect of egg jelly components on sperm physiology, extracellular environment, role of calcium and bicarbonate in sperm physiology, and physiological changes after spermiation (Krapf et al. 2011). A recent study has reported on the effect of extracellular conditions (i.e., exposure to water, differing temperatures) on sperm motility and structural properties (i.e., morphology, DNA integrity) collected from hormonally stimulated *Atelopus zeteki* (Della Togna et al. 2018). The study found that sperm longevity and its DNA integrity depends on the hypo-osmolality of the environment but not the temperature or hormonal stimulation method.

Spermatozoa can be preserved via the refrigeration of whole carcasses at 4 °C for later (up to seven days) postmortem collection of testicular sperm (Shishova et al. 2013). Viability has been demonstrated via the production of embryos, although the length of sperm viability appears to be species-dependent. Testicular sperm gathered from carcasses of *Rana temporaria* refrigerated at 4 °C could produce viable offspring via *in vitro* fertilization (IVF) for up to 6 days, while approximately 90% of sperm from carcasses of *Bufo baxteri* lost motility after

Table 2. Recovery of viable previously frozen sperm from specific anuran and urodelan families and successful IVF using previously frozen sperm. NOTE: Asterisk (*) denotes short-term refrigeration storage of spermatozoa at +4 °C, rather than cryopreservation.

Order	Family	Species	Recovery of viable frozen-thawed sperm	Successful IVF using frozen-thawed sperm
ANURA	Bufonidae	*Anaxyrus* (*Bufo*) *americanus*	Beesley et al. 1998	
		Bufo (*Rhinella*) *marinus*	Browne et al. 1998; Proaño and Pérez 2017	Browne et al. 1998
		B. bufo	Kaurova et al. 2008	Kaurova et al. 2008
	Hylidae	*Litoria peronii*	Browne et al. 2002a	
		L. brevipalmata	Browne et al. 2002a	
		L. fallax	Browne et al. 2002a; Upton et al. 2018	Upton et al. 2018
		L. nasuta	Browne et al. 2002a	
		L. latopalmata	Browne et al. 2002a	
		L. dentate	Browne et al. 2002a	
		L. phyllochroa	Browne et al. 2002a	
		L. lesueuri	Browne et al. 2002a	
		L. subglandulosa	Browne et al. 2002a	
	Leptodactylidae	*Eleutherodactylus coqui*	Michael and Jones 2004	
	Myobatrachidae	*Limnodynastis peronii*	Browne et al. 2002a	
		Crinia signifera	Browne et al. 2002a	
		Philoria sp.	Browne et al. 2002a	
	Pipidae	*Xenopus laevis*	Sargent and Mohun 2005; Mansour et al. 2009; Pearl et al. 2017	Sargent and Mohun 2005; Mansour et al. 2009; Pearl et al. 2017
		X. (*Silurana*) *tropicalis*	Sargent and Mohun 2005; Pearl et al. 2017	Sargent and Mohun 2005; Pearl et al. 2017
	Ranidae	*Rana temporaria*	Kaurova et al. 1996; Kaurova et al. 1997; Mansour et al. 2010; Shishova et al. 2011	Kaurova et al. 1996; Kaurova et al. 1997; Mansour et al. 2010; Shishova et al. 2011
		R. sylvatica	Mugnano et al. 1998; Beesley et al. 1998	
		R. pipiens	Beesley et al. 1998	
		Pelophylax lessonae	Uteshev et al. 2013	Uteshev et al. 2013
URODELA	Cryptobranchidae	*Andrias davidianus*	Peng et al. 2011	
		Cryptobranchus alleganiensis bishopi	Unger et al. 2013	
		C. alleganiensis alleganiensis	Nashville Zoo Hellbender Conservation 2018	Nashville Zoo Hellbender Conservation 2018
	Ambystomatidae	*Ambystoma tigrinum*	Marcec et al. 2014; Marcec 2016	Marcec 2016
		A. mexicanum	Figel 2013	
	Salamandridae	*Pleurodeles waltl*	Uteshev et al. 2015*	

36 hours, and no motility was present in sperm of *Andrias japonicas* after only two days (Roth and Obringer 2003). Healthy and reproductive adults that have produced offspring has been achieved via cryopreservation of sperm (Pearl et al. 2017; Upton et al. 2018; Table 2). Three primary factors affect the success of amphibian sperm cryopreservation, including: 1) cryoprotectants used, 2) sampling and acclimation of sperm to cryoprotectants, and 3) freezing rates (Browne and Figiel 2011). Specific published protocols and references for cryopreservation of sperm and IVF of 28 species and subspecies are listed in Table 2.

Spermic urine or urinal sperm, the cloacal urine containing a suspension of seminal plasma and mature

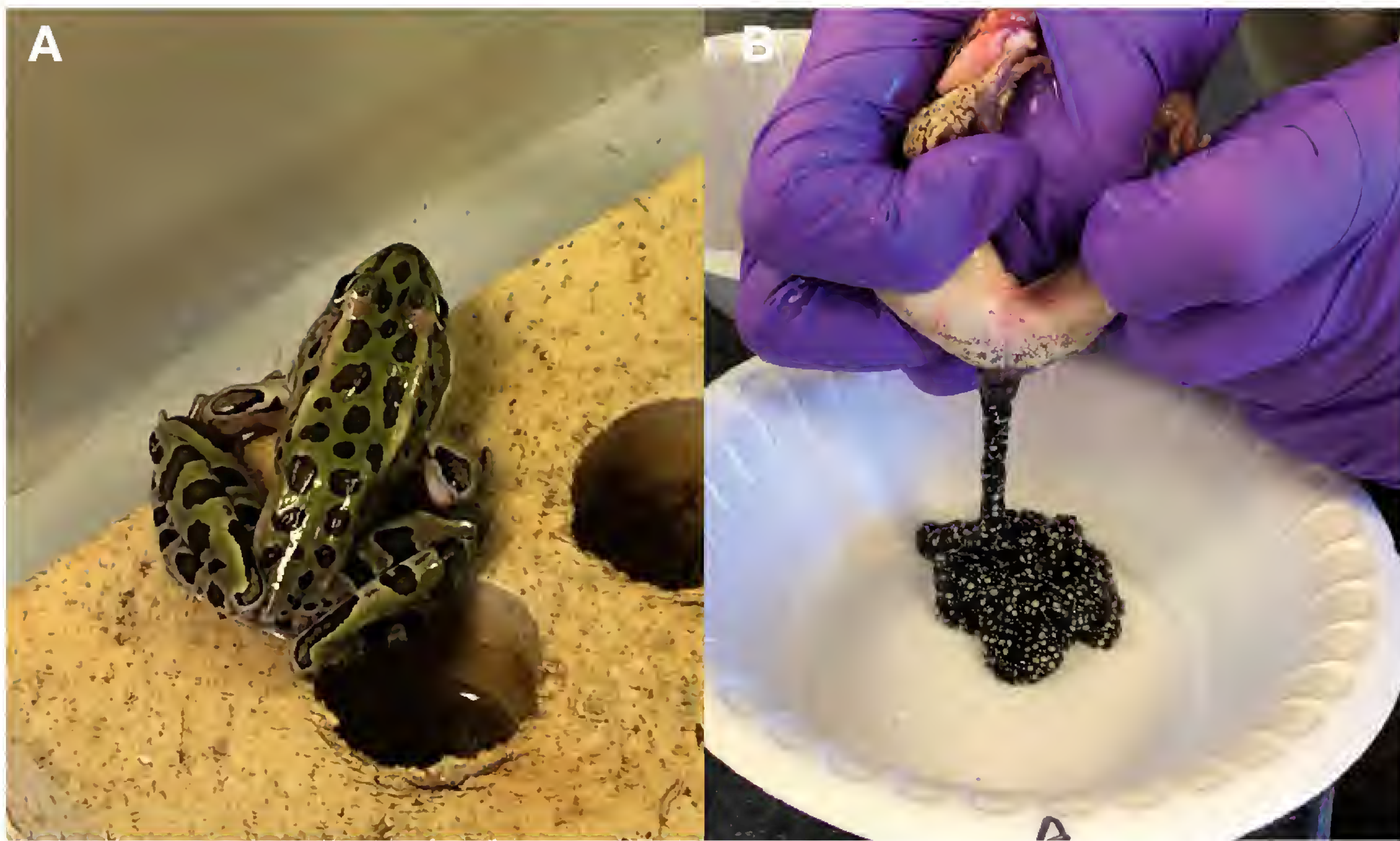

Fig. 2. Procedures used to obtain amphibian eggs or sperm for use in ARTs. A) Gravid female Leopard Frog (*Lithobates* sp.) after gonadotropic hormone injection. **B)** Expressing eggs into container by pressing on abdomen and pushing thumb toward cloaca; eggs can be fertilized (i.e., IVF) by fresh or cryopreserved sperm. Sperm can similarly be released from males by pushing towards the cloaca and releasing sperm naturally (in season) or after injection of gonadotropic hormones (e.g., HIS).

sperm, can be a source of gametes that eliminates the need to sacrifice live frogs. Spermic urine can be induced from males through the intraperitoneal administration of 50 micrograms of Luteinizing Hormone-Releasing Hormone analog (LHRHa) and manual massage of the area between the bladder and cloaca to induce urination (Fig. 2 demonstrates similar technique for manual release of eggs; Ananjeva et al. 2017; Kouba et al. 2012, 2013; Shishova et al. 2011). Cryopreservation of hormonally-induced sperm have been successful; concentrations of 200 × 106/mL are mixed in a 1:1 ratio with cryodiluents (e.g., glycerol, DMSO, sucrose) to form cryosuspensions with concentrations of 15 × 106/mL of HIS to achieve the highest fertilization percentage (Shishova et al. 2011). More recently, sperm has been collected from hormonally stimulated *Atelopus zeteki* by Della Togna et al. (2018) following intraperitoneal injection of gonadotropin-releasing hormone (GnRH) agonist (4 mg/g of body weight), hCG (10 IU/gbw), or Amphiplex™ (10 mg/gbw metoclopramide hydrochloride; 0.4 mg/gbw).

Salamander Sperm Collection and Preservation

The majority of salamander species have internal fertilization with males laying spermatophores. Sperm has been successfully collected from salamanders after sacrificing males and isolating the ducti deferens of *Pleurodeles waltlii*, *P. poireti*, and *Cynops pyrrhogaster* (Jaylet and Ferrier 1978; Watanabe et al. 2003). Spermatophores have also been collected from an internally fertilizing salamander, *Ambystoma mexicanum*, using two cryodiluents: 10% sucrose solution and Simplified Amphibian Ringers (SAR); SAR proved better at recovering more active sperm (Figiel Jr 2013). Urinal sperm has also been isolated as a suspension of spermatozoa for use in ARTs (Mansour et al. 2011; Uteshev et al. 2015). *In vivo* methods of obtaining sperm have been developed for *Ambystoma mexicanum*, *Andrias davidianus*, *Cryptobranchus a. alleganiensis*, and *Pleurodeles waltlii* (Browne and Figiel 2011; Mansour et al. 2011; Uteshev et al. 2015). Cryopreservation of the sperm of *Ambystoma tigrinum* has also been successful with subsequent fertilization achieved via IVF, although no embryos survived passed the neurula stage (Table 2; Marcec et al. 2014; Marcec 2016).

Caecilian Sperm Collection and Preservation

Caecilians are the only order of amphibians that use internal insemination. Sperm has been obtained from *Uraeotyphlus narayani* by removing lobes of the testis, washing them thoroughly in amphibian physiological saline solution (pH 7.4), and macerating them (George et al. 2005). This study demonstrated that sperm are motile when released from the testis, not requiring post-testicular physiological maturation. In addition, the secretory material of the Müllerian gland contributes to enhancing the speed and duration of motility of the spermatozoa. Although no known cryopreservation studies exist for this group, future development of protocols is encouraged, especially for threatened species.

Oocyte/Embryo Collection and Preservation

The collection of oocytes from live frogs can be achieved by natural collection via spawning or via artificial manipulation by stripping, excision from the ovaries, or through the use of hormones to stimulate release (Kouba et al. 2012). Natural collection through spawning can be successful but may require that animals are collected in the field during specific periods of the reproductive cycle or housed for periods of time until natural spawning occurs (Figiel Jr 2013; Fitch 1970). Manually extruding oocytes by pushing on the abdomen towards the cloaca with the fingers can greatly decrease experimental procedural time (Fig. 2). Amplexing males may fertilize oocytes that have spawned spontaneously, or IVF can be completed. The size of the spawning enclosure (e.g., plastic box) and depth of the simplified amphibian Ringer's (SAR) depends on the general size of the species and if artificially or naturally fertilized oocytes will require more solution to enable IVF (Browne and Zippel 2007). Newer methods for obtaining oocytes *in vivo* include induction of females using gonadotropic hormones and use of a glass rod to extended the cloacal sphincter, allowing release of oocytes (Kouba et al. 2012, 2013). Eighteen species of frogs, including thirteen genera, and two salamander species of two different genera, have successfully oviposited after hormone induction to date (Calatayud et al. 2017). For a review of attempts to induce oviposition using hGH in anuran (n = 21, including *Mixophyes fasciolatus*) and urodela (n = 6) species see Clulow et al. (2012). Manually stripping ooctyes through palpation may be possible for some species if females ovulate but do not spawn (Fig. 2; Whitaker 2001). Although effective in some groups (i.e., ranids), manually stripping oocytes is generally not suitable for those species with egg masses produced as paired strings (e.g., toads; Browne and Figiel 2011).

Stimulated release of anuran and urodelan ooctyes is commonly achieved using hCG, LHRHa, or GnRH (Ananjeva et al. 2017; Clulow et al. 2018; Uteshev et al. 2015). For those groups for which current protocols have not yet been successful, an improved suite of tools, including access to pure or recombinant endogenous gonadotropins, are likely needed (Clulow et al. 2018). Although pituitary gland suspension is used commercially with common species to stimulate oogensis, it is no longer recommended for use with threatened species since pituitary tissue may transmit pathogens (Ananjeva et al. 2017).

The collection of oocytes post-mortem for use in IVF was first reported by Dabagyan and Sleptsova (1975) for anurans and by Bordzilovskaya and Dettlaf (1975) for salamanders with mature oocytes being excised from the lower part of the oviduct after ovulation (Ananjeva et al. 2017). See Dettlaff and Vassetzky (1991), Dabagyan and Sleptsova (1991), and Bordzilovskaya and Dettlaf (1991) for English translations. A recent study using *Rana temporaria* has shown that oviductal oocytes can be stored for up to five days in carcasses refrigerated at 4 °C or when isolated from the oviduct, leading to 70% normal development (Ananjeva et al. 2017; Uteshev et al. 2018). The refrigeration of oocytes can be a simple but critical technique that allows gamete transport when collected from field populations or between institutions involved in ARTs. In addition, the period that oocytes remain fertile can be increased by lowering temperature to reduce metabolism or increasing osmolarity to slow oocyte gel coat hydration (Browne et al. 2001).

The cryopreservation of neither mature oocytes nor embryos has yet been achieved. Oocytes are likely difficult to cryopreserve due to their high cellular fat content, size, shape, and low permeability characteristics leading to cell damage during freezing (Lawson et al. 2013). Although oocytes have not yet been cryopreserved for future use, they can be collected for use in ARTs, and can remain viable and fertilized up to as long as 30 days (Uteshev et al. 2018). A number of techniques appear promising *in lieu* of developing a method for cryopreserving oocytes or embryos, including cryopreservation of blastomeres used in conjunction with somatic cell nuclear transfer (SCNT) and androgenesis with frozen spermatozoa (Clulow and Clulow 2016). A recent discovery that coral larvae can be cryopreserved through vitrification and thawed to resume swimming after laser warming provides great promise that breakthroughs in other taxonomic groups may lead to the successful cryopreservation of amphibian oocytes and embryos (Daly et al. 2018).

In Vitro Fertilization (IVF)

The terms artificial fertilization (AF) and IVF are both used in the literature and by amphibian reproductive biologists to denote the artificial insemination of eggs in a Petri dish. Kouba and Vance (2009) suggested that IVF is more appropriate for salamanders and caecilians given they exhibit internal fertilization, and AF is a more appropriate term for anurans where external fertilization is more common. For clarity, we have chosen to use the term IVF to denote when fertilization is performed manually by a researcher. IVF of frog oocytes have been performed for decades in experimental embryology (Rugh 1962; Dabagyan and Sleptsova 1975). The general procedure, which can be used for all anuran species, involves placing 20–50 oocytes with 200–500 µl of sperm (urinal or testicular) in a Petri dish. The fertilized eggs are washed with fresh water and left to develop; successful fertilization is identified via cleavage approximately 3–8 hours later, although species may vary in developmental timing and rates (Ananjeva et al. 2017).

Although most salamanders have internal fertilization, IVF has been successful for some species. Gametes obtained post mortem from *Ambystoma mexicanum* were first used in IVF of salamander oocytes (Brunst 1955). More recently, Mansour et al. (2011) fertilized oocytes

using gametes sampled from the same species *in vivo* with spermatozoa from semen obtained through hormonal induction and abdominal massage. In 2012, the Nashville Zoo in the U.S. announced the first successful captive breeding of Eastern Hellbenders (*Cryptobranchus a. alleganiensis*) from eggs produced and artificially fertilized from captive zoo animals, and later in 2015, the zoo was successful in hatching a salamander from an egg that was artificially fertilized with cryopreserved sperm (Nashville Zoo Hellbender Conservation 2018).

Tissue Preservation for Cell Lines

Viable fibroblast cell lines are one of the most versatile genetic resources and can play an important role in *ex situ* conservation. Cell lines banked in LN_2 can be maintained indefinitely and provide a continual source of genetic material for a wide variety of purposes (Ryder and Onuma 2018). These cells can be utilized to obtain chromosomes, expanded to generate large quantities of DNA/RNA, and used for SCNT because they are living and dividing (Houck et al. 2017). Induced pluripotent stem cells (iPSCs) capable of differentiation into multiple cell types have been generated from skin cells of mammals, including humans, mice, and rhesus monkeys, by direct molecular reprogramming (see Houck et al. 2017 for review). More recently these methods were successfully applied to cryopreserved adult fibroblasts of endangered mammal species, including a primate (*Mandrillus leucophaeus*) and the northern white rhinoceros (*Ceratotherium simum cottoni*; Ben-Nun et al. 2011; Korody et al. 2017). Preserving amphibian genetic material as fibroblast cells while populations are still available will allow the greatest number of options for future genetic rescue when methods have been adapted for non-mammalian species. Once a population is reduced to a critically small number of individuals the feasibility of successfully establishing a significant number of cell lines diminishes.

Post-mortem tissue and organ samples should be collected while they are still viable (not necrotic or frozen), and, at a minimum, cryopreserved using DMSO so that fibroblast cell lines can be established and cryopreserved in genetic resource collections at a later date. Freezing tissue biopsy samples for later initiation of cell culture (i.e., "tissue piecing," Fig. 3) is described in Houck et al. (1995), Gamble (2014), and Houck et al. (2017, protocol 24.11); herein we summarize this method. Tissue is collected in vials containing cell culture media with antibiotics and held at 4 °C (or room temperature if refrigeration is not available); ideally tissues are stored in media for less than three days but can potentially be stored up to 10 days if no contamination occurs. Under aseptic conditions tissue is then minced into one mm³ fragments and placed in cell culture medium containing 10% DMSO and either transferred to a primed LN_2 dry shipper for short-term storage during transport or placed directly into a long-term LN_2 storage. Tissue prepared this way

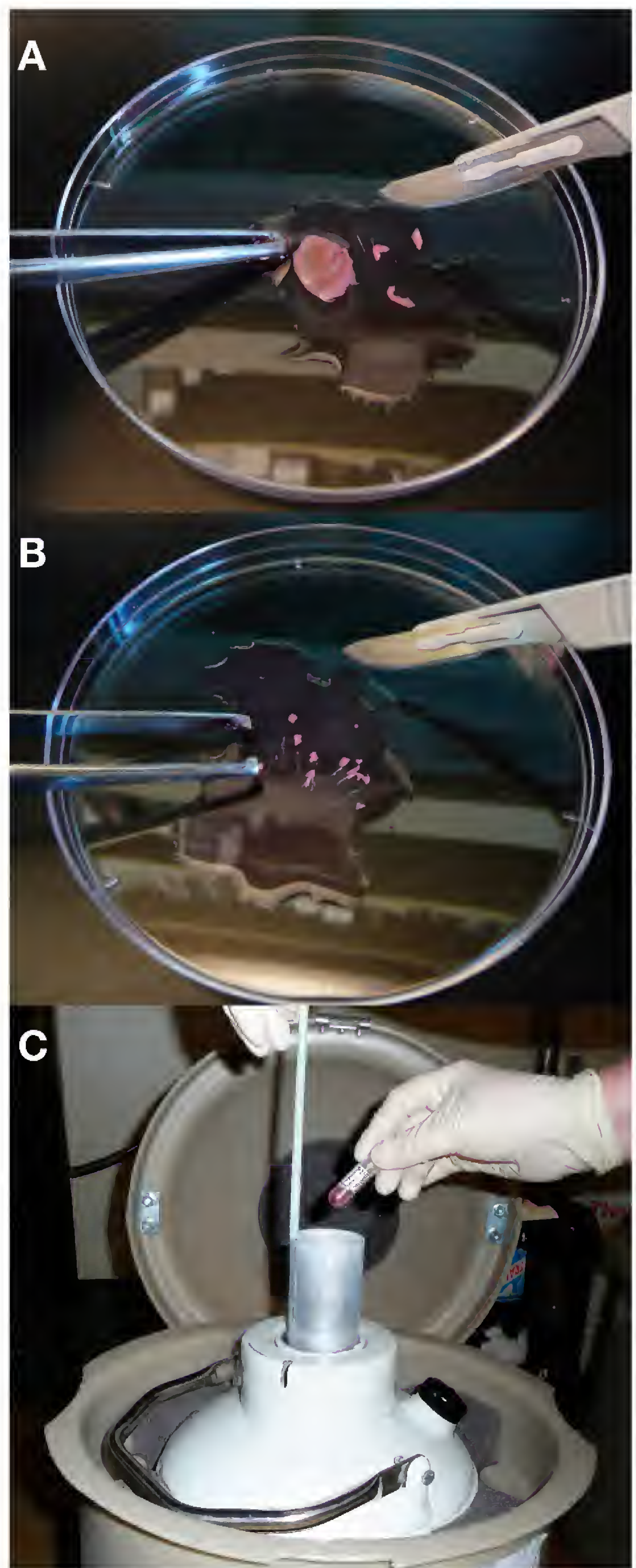

Fig. 3. The "tissue piecing" protocol used to preserve viable cells for establishment of cell lines in the future. A) Tissue is cut into long, thin strips. **B)** Tissue is diced into 1 mm^3 fragments before adding medium containing 10% DMSO as a cryoprotectant. **C)** Prepared tissue is stored in LN_2 until future cell culture is possible; those without cell culture capability can transport samples using a dry shipper to maintain cold-chain.

and kept in LN_2 can be stored indefinitely and later transported to a lab with experience in tissue culture to estab-

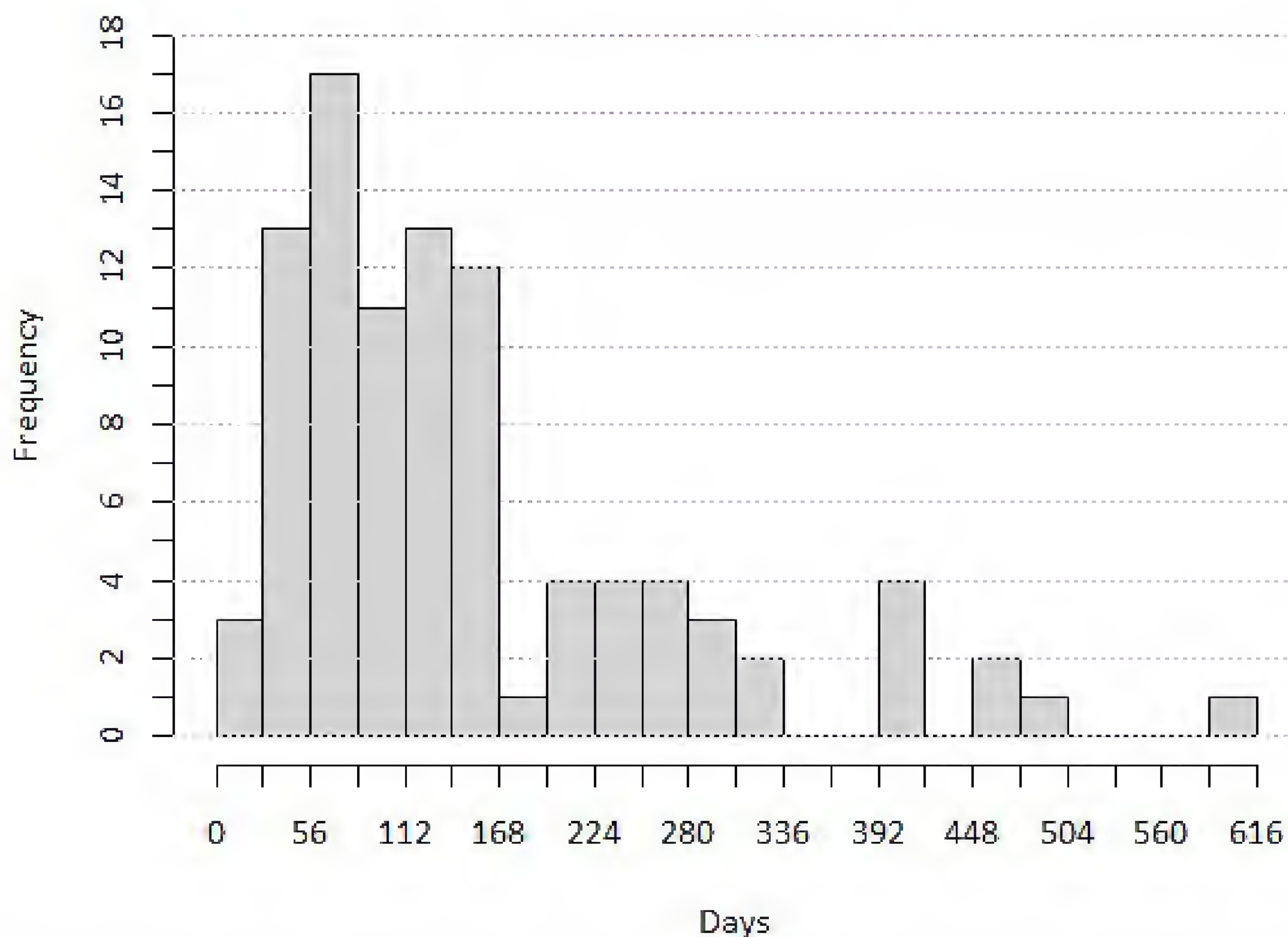

Fig. 4. Length of time from cell culture initiation to freezing for amphibian cell lines in San Diego Zoo's Frozen Zoo®. Low = 19 days; high = 596 days; average = 154 days.

lish cell lines. The preferred amphibian tissues to collect for this method (in order) include: whole limb (i.e., foot), tongue, skin, and gonads. Other tissues that have been successfully used to establish amphibian cell lines include: eye, tail (juveniles), whole tadpoles, and kidney.

Challenges Associated with Amphibian Cell Culture

A number of challenges are associated with the establishment of amphibian cell lines, demonstrated by the fact that few biobanks contain cell lines, and most of these hold mammalian, avian, and reptilian cells. Mammalian cell culture is generally successful following the many well-described methods, including Freshney (2005) and Masters (2003), but establishing cells from amphibian tissue has proven to be more challenging. Although there are many reports for methods of amphibian cell culture (see Okumoto 2001 for summary), there are very few known cryopreserved viable amphibian cell lines in collections. A literature search yields only a few papers that describe methods used to establish cell lines. The American Type Culture Collection (ATCC), a biological materials resource and standards organization, has limited amphibian cell lines (American Type Culture Collection 2017). The largest known collection of amphibian cell lines is curated at the San Diego Zoo's Frozen Zoo® (Chemnick et al. 2009; San Diego Zoo Institute for Conservation Research: Frozen Zoo® 2018), currently containing 95 cell lines from 83 individuals and 21 species. Some of the important parameters that were identified for the success of cell lines established in the Frozen Zoo® collection include: media similar to that used for mammalian cells, a low oxygen environment, incubation temperatures of 20–23 °C (for taxa from cool climates) or 27–30 °C (for tropical species), and use of the explant method instead of enzymatic digestion (Houck et al. 2017; Houck, unpubl. data).

Contamination is a greater challenge in amphibians than in other groups, such as mammals, in part because many amphibians dwell in moist environments. Using antibiotics and antimycotics, such as penicillin, streptomycin, gentamicin, normocin, and fungizone (amphotericin B), is a crucial part of sample collection and tissue culture in this group. Even with use of these widely effective antibiotics, one of the most common causes of failure in amphibian cell culture is contamination. Keeping wild-caught individuals in captivity for several weeks may also reduce contamination (Tony Gamble, pers. comm). The other common failure associated with amphibian cell culture is absence of cell growth. This can be attributed to poor sample quality (i.e., few viable cells to begin with), and sub-optimal growth conditions, such as temperature and media. Optimal conditions vary by species (as was noted for sperm collection/preservation previously), and for most species these conditions are not yet known. One of us (MH) has found that methods and conditions that are successful for some species grown in the tissue culture lab can fail to work on other species, sometimes even those in the same genus.

Methods for amphibian cell culture have not yet been fully optimized, and the process takes an average of more than 150 days in culture before a sufficient number of

cells can be frozen (Fig. 4). This period of time is significantly longer when compared to an average of 21–28 days for mammalian cells (data from San Diego Zoo tissue culture lab). Cells that are in culture for long periods of time are prone to chromosomal changes, and this has been noted in long-term culture of amphibian cells where the modal diploid number is observed to differ from expected diploid numbers based on chromosome numbers derived from short-term cultures, such as blood or bone marrow (Okumoto 2001; Houck, unpubl. data). Although there is a need for further improvements in amphibian cell culture methods, recent advances have led to the addition of over 80 cell lines to the Frozen Zoo® and suggest that widespread success across the community is possible. Researchers working with these methods will likely be able to enhance the protocols further, leading to shorter culture times and broader application to other amphibian taxa. Until these issues are resolved, freezing tissues with DMSO using the "tissue piecing" method previously described is recommended for those who have access to post-mortem amphibians (Fig. 3; Houck et al. 1995; Gamble 2014; Houck 2017, protocol 24.11). This procedure allows researchers to preserve samples for future initiation of cell culture, which provides time for the improvement of methods, safeguards valuable or rare samples until methods are more successful for specific species, and allows those with experience in cell culture (possibly at a collaborating institution) to propagate the cell lines.

Captive Breeding Programs

Captive breeding is an important aspect of amphibian conservation as it ensures the survival of species that cannot be safeguarded in their natural habitat. It is also often the only way to collect oocytes of specific species since they cannot currently be cryopreserved with current methods. The Amphibian Ark (AArk) focuses on *ex situ* programs for species that cannot currently be safeguarded in the wild, and their survival is dependent on conservation breeding programs (Amphibian Ark: Establishing *Ex Situ* Amphibian Programs 2018). AArk is convinced that two steps are vital to executing a successful *ex situ* conservation program, in particular if release back into the wild is required: 1) the program must be completed entirely within the range country, and 2) the population must be maintained, housed, and confined separate from populations outside its range. Facilities located within the species natural range that exclude non-native species are examples of best practice, thereby requiring the smallest budget and least amount of effort to be successful (Pessier and Mendelson 2017). Conservation breeding programs or survival assurance colonies intending to reestablish amphibians into their natural habitat should manage animals in perpetual isolation (e.g., committed buildings or rooms) separated from amphibians originating outside of the species native range (Amphibian Ark: Establishing *Ex Situ* Amphibian Programs 2018). Long-term quarantine of amphibians is also required for animals outside the natural range of species that will be subsequently translocated or reintroduced (Pessier and Mendelson 2017). Mixed collections or those "cosmopolitan" in nature (e.g., facilities housing animals from multiple geographical) pose an increased risk of introducing infectious diseases to natural populations in reintroduction and translocation programs. As a result, these housed animals may be exposed to diseases not already exhibited in the controlled population and potentially spread them to wild populations. The ideal situation and lowest risk position for introducing pathogenic diseases to native amphibian populations within reintroduction programs is, therefore, when conservation assurance colonies are positioned safely within the native country of the amphibian species or species group, and the conservation breeding facility maintains only species from within the species native territory or country (Pessier and Mendelson 2017). Lastly, the use of dedicated equipment and tools, committed or single purpose footwear, personal protective equipment (e.g., lab coat), and workflow methods that diminish risk of introducing non-native pathogens into amphibian aggregations should be enforced as a top priority.

Ex situ conservation efforts focused on amphibian species can be hampered by inadequate or incomplete knowledge of presumptive animal species. It is, therefore, recommended that a phylogenetic analysis of the wild species from its natural habitat be completed before conservation breeding efforts begin to ensure that cryptic species do not remain unidentified (Yan et al. 2018). Crawford et al. (2013) used DNA barcoding of the COI and 16S genes to review mitochondrial diversity in captive communities of ten species of amphibians from the Neotropics managed as an *ex situ* assurance plan. Substantial cryptic genetic variation was identified within three of ten *ex situ* populations, and three other species exhibited cryptic diversity in natural indigenous populations but not in captive populations. DNA barcoding can provide the first method to identify cryptic diversity, but an intergrative taxonomic approach that uses data from multiple sources, including molecular data, morphology, ecology, and advertisement calls, should ultimately be used for species delimitation (Vieites et al. 2009; Evans et al. 2015).

The Future of Species Conservation

Integrating the current practices for the preservation of amphibian genetic resources and living tissues will ultimately aid in both basic research and the practice of species conservation. We, therefore, have devised two decision trees to allow researchers to determine which type(s) of samples that they can preserve both for research and amphibian conservation efforts (Fig. 5, 6). These two workflows are distinct, depending on whether

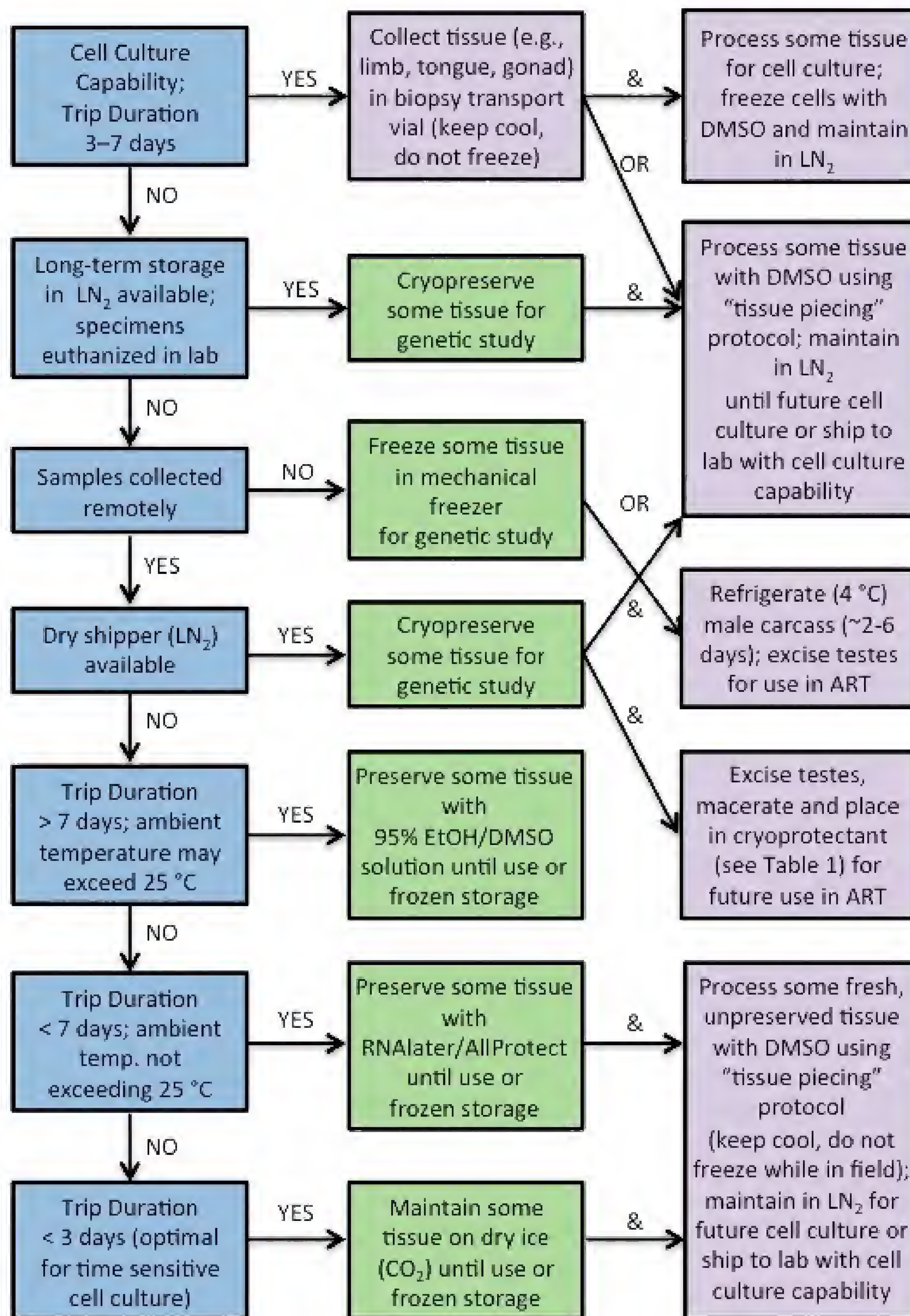

Fig. 5. Decision tree used for specimens euthanized to obtain tissue. Blue indicates steps in the decision tree. Green indicates procedures that will lead to preservation of tissues for genetic study. Purple indicates procedures that lead to achieving multiple goals, including cell culture and obtaining gametes for current or future ART. NOTE: Breeding and artificial fertilization can result in offspring that can be used for genetic purposes, thereby achieving multiple goals.

animals will be euthanized (e.g., museum specimens) or tissues are being collected from live animals. Researchers can thereby determine whether they may be able to additionally obtain and preserve gametes and/or cell culture tissues for immediate use in ARTs or long-term cryopreservation given the laboratory or field conditions and available equipment. We believe that these decision trees will allow researchers to more easily integrate current practices for the preservation of amphibian genetic resources and living tissues. We also hope that these resources will aid in the development of best practices for species conservation in assisted reproductive technologies.

Researchers can maximize the downstream research potential of the sample via their selection of preservation method(s), ultimately allowing the broadest range of future uses in both basic research and species conservation. Regardless of the study goals, researchers should care-

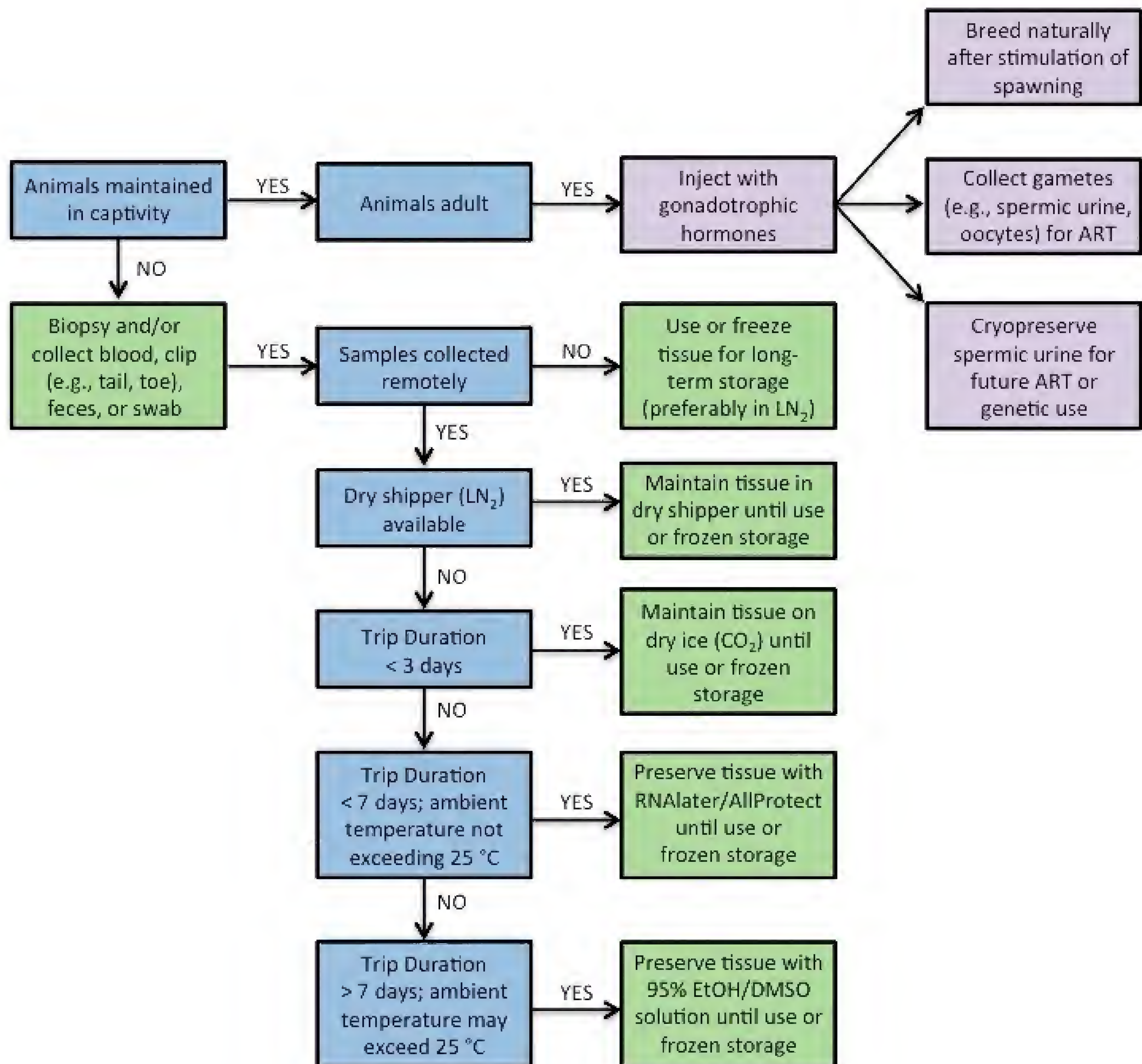

Fig. 6. Decision tree used to obtain tissue from live animals. Blue indicates steps in the decision tree. Green indicates procedures that will lead to preservation of tissues for genetic study. Purple indicates procedures that lead to achieving multiple goals, including obtaining gametes for current or future ART. NOTE: Breeding and IVF can result in offspring that can be used for genetic purposes, thereby achieving multiple goals.

fully weigh their choices and consider the conditions associated with the collection of their genetic resources as poor preservation can hinder genetic analyses and render samples useless. Tissue sampling methods and standards for vertebrate genomics have been proposed that include four categories for classifying the utility of tissues and DNA being prepared for Genome 10K (G10K) and other similar projects (Wong et al. 2012). The authors suggest that researchers attempt to collect more high-quality samples that include: sufficient flash-frozen tissue or immediate extraction of DNA for a minimum of one mg of DNA, multiple tissues for RNA sequencing and transcriptome analysis, and viably frozen tissue pieces suitable for establishing cell lines. If the standard collection of genetic resources for molecular analyses includes the collection of more high-quality samples, future use may include species propagation.

The standardization of pre-analytical variables by biobanks is also critical for understanding downstream sample quality. The Standard PREanalytical Code (SPREC) was developed to provide a comprehensive and practical tool to document preanalytical (e.g., collection, processing, storage) biospecimen data (Lehmann et al. 2012). Biospecimen Reporting for Improved Study Quality (BRISQ) are additional standards that have been proposed for information that should be reported about biospecimens in scientific publications and regulatory submissions (Moore et al. 2011). Application of quality management systems (e.g., SPREC, BRISQ) should be applied to biobanks working with animal genetic resources, but they must first be reviewed and adapted to ensure that they capture the broad range and diversity of non-human samples (Benson et al. 2016).

WAZA recently approved a resolution calling for ac-

celerated efforts to protect biodiversity and aid species conservation by establishing and biobanking viable cell lines from tissue (Oliver Ryder, pers. comm.). If the collection and deposition of tissues preserved for eventual cell culture can become routine practice among researchers already preserving genetic material, particularly herpetologists working in the field, more cell lines may be biobanked in the future to aid in this effort. Collecting different tissue types or using specific methods may not be possible for those without the necessary equipment (e.g., dry shippers) or associations with genetic resource collections that can store the samples long-term; however, it may require minimal change for others. Establishing cell lines requires a high skill level and fairly specialized labs, but freezing tissues with DMSO using the "tissue piecing" method to allow the establishment of cell lines in the future is feasible when amphibians are already being euthanized for museum specimens or genetic analysis (Fig. 3; Houck et al. 1995; Gamble 2014; Houck 2017, protocol 24.11). An increasing number of biobanks associated with natural history collections are already cryopreserving DNA and RNA samples, so the addition of tissues processed using methods suitable for future cell culture may not be arduous. The deposition of amphibian tissue and cell lines as a standard practice in research projects worldwide (e.g., academic, government, non-governmental agencies, industry) may also facilitate future inter-institutional research and collaboration regarding cryopreservation and ARTs. Cell lines could potentially be reprogrammed into stem cells in the future, and their pluripotent nature could allow them to differentiate into gametes, such as sperm and ova. There is expanded potential for SCNT to create clones beyond what has been achieved (Gurdon 1962; Branco 2015) and potentially further conservation efforts. Thus far, fertile adults may be generated if donor nuclei are obtained from early embryos (Gurdon 1962). Neither of these methods (SCNT and developing cells lines as potential generation of gametes) are completely developed and achievable for potential use in species conservation, but they may only be attempted for genetic rescue when it becomes possible if species are preserved either in the wild or with biobanked cell lines.

Collaboration and Integration of Biobanks Globally

A number of programs have been initiated to collect the world's amphibian species that are threatened with extinction. The goal of a project spearheaded by the Amphibian Survival Alliance and Amphibian Specialist Group is to create a historically permanent record and resource (publicly accessible in sustainable repositories) of bioinformatics and tissue for amphibian species conservation and research. To that end, two target areas have been identified: bioinformatics of amphibian genomes and biodiversity preservation of tissues representing all amphibian species. The latter aims to cryopreserve tissues, develop cell lines, and promote ARTs for amphibian species, particularly those in immediate danger of going extinct and those found in highly endangered habitats. *Amphibia Bank* is a collaborative effort that has been proposed as a means to bring together cell culture and tissue repositories and promote the collection of cell samples (e.g., blood, cell cultures, tissues, and spermatozoa with the potential to include eggs and embryos in the future; Lawson et al. 2013). This project aims to represent every amphibian species on earth in participating biobanks but will first focus on collecting threatened and endangered species and representatives of every genus. An initial pilot project for this effort involves the collection of all North American salamander species, which was identified as critical because of the spread of the pathogenic chytrid fungus *Bsal* (Gray et al. 2015; Hassapakis and Clark 2017).

Regional and international research collaborations between zoos/aquariums, natural history museums, and other academic institutions (e.g., universities, colleges) offer a unique opportunity to move amphibian research and conservation forward. Although research collaborations may have existed between these institutions, including the accession of specimens originating from zoos into natural history museums, modernization of these relationships may include new partnerships between biobanks. Natural history museum collections may hold specimens that reflect historic distributions and former variation in fragment populations, which may be useful information for conservation programs at zoos/aquariums. Zoos and aquariums are often focused on public education, but they are unique in their capacity to develop and sustain long-term projects through fundraising efforts and dedicated staff. In addition, zoos may have material that is poorly represented in museum collections, including endangered species, which can be used by natural history museums in research regarding aging, anatomy, functional morphology, pathology, reproductive biology, and taxonomy (Kitchener 1997). Formalizing partnerships between zoos/aquariums, natural history museums, universities, and other institutions, including signing Memoranda of Understanding, would increase and improve collaboration in areas of common interest.

Collaboration among academic institutions should make efforts to include input and participation from additional stakeholders that make up the biobanking community. One such proposal has been put forth to establish a genome resource bank (GRB) for threatened Australian amphibians (Mahony and Clulow 2005). The major objectives of this GRB include: 1) captive husbandry to prevent species extinction, 2) maintenance of genetic diversity, 3) reduction of the number of individuals held in captivity, thus extending resources for a more diverse collection of species, and 4) selection for resistance to *Bd*. Progress has also recently been made in the United Kingdom (U.K.) with the CryoArks project, which was

funded by the Biotechnology and Biological Sciences Research Council (BBSRC) to increase access, organization, and species coverage in U.K. animal biobanks by providing infrastructure and expertise (U.K. Research and Innovation 2018). This biobanking project joins together various stakeholders, including Cardiff University, the Natural History Museum, National Museums Scotland, Royal Zoological Society of Scotland's Edinburgh Zoo and Highland Wildlife Park, University of Nottingham, and University of Edinburgh. CryoArks will also partner with the Frozen Ark Project and the European Association of Zoos and Aquaria (EAZA) whose biobanks focus on endangered species and zoo/aquarium animals, respectively. The first phase of the project will concentrate on aggregating genetic resources from the collaborating institutions to make the material discoverable and accessible, but future goals include the cryopreservation of living cells (Jacqueline Mackenzie-Dodds, pers. comm.).

Worldwide efforts focused on amphibian biobanking should target the most biodiverse countries (e.g., Brazil, China, Colombia, Democratic Republic of the Congo, Ecuador, India, Indonesia, Madagascar, Malaysia, Mexico, Papua New Guinea, Peru, Philippines, South Africa, United States, and Venezuela). High priority should also be given to threatened habitats (e.g., Madagascar, Borneo, Micronesia, Polynesia, Mediterranean Basin, Tropical Andes) and animal groups with large numbers of critically endangered species and unique species. Some examples of specific groups to be targeted include the genera *Atelopus*, *Pseudophilautus*, *Craugastor*, *Litoria*, *Mixophyes*, *Pristimantis*, *Plectrohyla*, *Rhinoderma*, *Andrias*, and *Cryptobranchus*, the Madagascan frog family Mantellidae, and monotypic families such as *Nasikabatrachus sahyadrensis* (Biju and Bossuyt 2003). ARTs require viable cells, thus the collection of "living tissue" (e.g., gametes, cell lines, and other tissues) is needed for future use. These collection efforts and technologies can no longer be applied as small-scale, final attempts (Clulow et al. 2014). The collection and cryopreservation of these living materials should be prioritized to include threatened and endangered species, as well as those from highly endangered habitats before they are no longer available. In conjuction with these efforts, the technologies needed for successful reproduction and the production of pleuripotent stem cells should be optimized.

Increasing capacity building and training, as well as sharing existing knowledge and technologies with institutions and scientists is essential to moving amphibian conservation efforts forward (Kouba et al. 2013). The continual improvement of best practices for biorespositories that incorporate practices and procedures specific to non-human biological samples also will aid in the improvement to the operation of biobanks associated with institutions such as natural history museums and zoos (Campbell et al. 2018; ISBER 2018). Symposia, workshops, online resources, and increased funding can all be used in education efforts, and the formation and interactions of research consortia aimed specifically at tissue collection, cryopreservation, and cataloging, allow additional opportunities for discussion and collaboration. Engaging researchers and staff in continents that do not yet have an active amphibian biobanking programs (e.g., Africa, South America, Central America, Asia) by establishing training opportunities will allow the transfer of knowledge and expertise needed to build stronger in-country networks. These in-country networks can ultimately aid in coordinating field biologists that are already documenting population declines and categorizing diversity to bank samples from animals in wild populations, which will greatly assist in these amphibian conservation efforts. The Global Genome Biodiversity Network (GGBN) was created to fill the need for a network of biobanks associated with natural history museums, herbaria, botanical gardens and other stakeholders, establishing a portal for locating samples that meet quality standards for genome-scale applications (Seberg et al. 2016). GGBN has formed task forces for important topics, such as data standards, policies, and biobank procedures, and brought together those working in both biobanks associated with natural history collections and zoos at annual meetings to compare and contrast methodologies. In addition, a tissue preservation study has been initiated that includes numerous GGBN member institutions to establish a clearer perspective on some of the most commonly used to preservative samples deposited in zoological and veterinary biobanks. The goal of the study is to increase standardization among animal biobanks and to enhance suitability of samples for current and future downstream analysis.

Conclusions

Biomaterials banked from amphibians are a vital resource that can only be acquired and developed while these resources exist, thus underpinning the importance of their collection now for both present and future uses. We expect that scientific advancements associated with the preservation of tissues and ARTs will be made as laboratory protocols and methodologies are more widely used for amphibians. It is critical that researchers stay up-to-date with new findings and apply best practices to maximize the potential of the valuable genetic samples that they collect. Technological developments associated with the long-term storage of tissue samples may also allow more institutions to build internal biobanks. These new biobanks should integrate into larger networks to aid in regional or global conservation efforts. Lastly, we hope that cooperation and research partnerships among stakeholders, as well as education and promotion within the scientific community, will lead to scientific progress in these areas to aid in amphibian conservation.

Acknowledgements.—We thank Julie Strand for manuscript feedback, as well as Julie Fronczek, Catherine Avila,

Ann Misuraca, and Marisa Korody for information regarding cell culture and karyotyping. Many thanks to Katy Thomson for assistance with Fig. 4. Thanks goes to Jaclyn Zelko, Chester Figiel, Jr., William Wayman, and the U.S. Fish and Wildlife Service Warm Springs Fish Technology Center for their friendship and collaborative research opportunities. A special thanks is extended to Terrence "Terry" R. Tiersch and for his invaluable friendship, kindness, expertise, and expanding his world view to include amphibians.

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